# General 3 kb Library Creation Protocol

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## Summary

This protocol is for the high-throughput production of subcloned BAC DNA libraries. The starting material is generally purified BAC DNA with an average length of 120 kb and the finished products are arrayed microtiter plates of subclones with an average length of 3 kb that are ligated into pUC18 vector and transformed into ElectroMAX DH10B competent cells.

This protocol may also be used, after minor modification, to subclone microbial genomes, as well as other larger whole genomes.

## Subcloning -- Materials & Reagents

| Materials/Reagents/Equipment | Vendor | Stock Number |
|---|---|---|
| *Disposables* | | |
| QIAquick Gel Extraction Kit (50) | QIAGEN | 28704 |
| or | | |
| QIAquick Gel Extraction Kit (250) | QIAGEN | 28706 |
| *Reagents* | | |
| T4 DNA Polymerase 1U/µl | Roche | 1004786 |
| DNA Polymerase I (Klenow) 5000U/mL | NEB | M0210L |
| Fast-Link DNA Ligation Kit | Epicentre | LK6201H |
| 10mM dNTP mix | Amersham | US77212 |
| pUC18 SmaI / SAP | Roche + JGI | 0885819 + JGI cut |
| *Equipment* | | |
| Agarose gel box and two 13 teeth combs | Owl | D-14 |
| HydroShear | GeneMachines | HSA-01-P |
| Standard HydroShear Shearing Assembly | GeneMachines | HSA-025 |
| HydroShear Syringe | GeneMachines | HSA-S |
| HydroShear Wash Kit | GeneMachines | HAS-kit-01 |

## Procedure

**Shearing:**

1. Vortex, and then Spin isolated DNA for 5 min @ 12,000 rpm.

2. Pipette 100 µl into a new micro centrifuge tube (be careful to not take from bottom of tube).

3. Shear 100 µl of ~30 ng/µl isolated BAC DNA (~3 µg total) with the HydroShear from Gene Machines.

4. Open the HydroShear program and set the shearing parameters as follows:
   a. DNA volume = 100 µl, # of Cycles = 25, Speed Code = 12 or 13 (qc each hydroshear for size).
   b. Wash Cycles: 3x with 0.2M HCl, 2x with 0.2M NaOH, 5x with filtered TE. (Use wash kit)

5. After shearing, collect the samples into micro centrifuge tubes.
   ***"Place on ice IMMEDIATELY after shearing"***
   The volume recovered after shearing should be approximately 95 µl.

**Blunt End Repair:**

1. End repair 95 µl of each sheared sample in a micro centrifuge tube.

2. To each sample, add:

| | **1 Rxn** | **13 Rxns** |
|---|---|---|
| 10x Klenow Buffer ---------- | 13 µl | 169 µl |
| 10mM dNTPs ---------- | 10 µl | 130 µl |
| T4 DNA Polymerase (1U/µl) --------- | 6 µl | 78 µl |
| Klenow Fragment (5000U/mL) ------- | 6 µl | 78 µl |
| | **35** µl | **455** µl |

   *A well mixed cocktail of the above can be prepared (***store on ice***) and aliquoted if needed.
   ***Add 35 µl of the end-repair master mix to each tube containing 95 µl of sheared DNA.

3. Close tube, Mix/vortex for ~3 sec.

4. Quick spin.

5. Allow the reaction to proceed at room temperature (on bench top) for 40 minutes.

6. After 40 minutes, heat inactivate the tubes in a hot block @ 70°C for 15 minutes, then 4°C (on ice) for 5 minutes. The tubes must be frozen at -20°C (freeze) for at least 30 min, or can be stored overnight @ -20°C.

**Agarose Gel Separation:**

1. Make one ~300 ml 1% TAE agarose gel (use 23 cm x 14 cm gel tray & two 13 tooth combs) for every 12 DNA samples. (load a sample every other well, total of six samples per comb)

2. Thaw the end-repaired samples and add 20 µl of a 30% glycerol 1x loading dye to each tube.

3. Load ~130 µl of each end repaired product into each well (**It's very important to keep track of sample name/order and to make sure of no cross contamination. Also, load 15 µl of lambda hindIII size marker (marker 2 only) into the left, middle, and right small wells. Run for ~60 min @ 100v. **It is very important that you get visible separation between the 2.0 and 2.3 kb size marker bands.** After this happens you can image and easily cut out the desired ~3-4 kb band for each sample.

4. Image gel (image for no longer than 5 sec. to minimize UV exposure) and save photo.

5. Cut gel into 4 parts (through the size marker in the middle of the gel & above each set of 10 wells. Cut out each gel fragment between the 2.2 kb & 4.3 kb size marker (~9.0 mm x 1.5 mm) using clean razor blade (each slice weighs ~180 ng). The thinner the band the more consistent the insert size will be. Put each slice into its own **well-labeled** 1.5 ml microfuge tube.
   ** Important to minimize UV exposure to yourself (use long sleeves & face shield) & to the samples (use laminated paper each section of samples not being cut). Turn off UV after cutting out each band.

6. Image gel after 3-4 kb band extraction and save photo.

**Gel Purification Filter:**

1. Use / follow Qiagen gel purification handbook. Heat QG buffer in ~60°C oven ~10 min before using. You can do this while you are cutting out each slice. This is not a requirement; it will just help with the melting of the gel slices. Add 600 µl of warm Buffer QG to each tube containing a gel slice, close top and mix by hand a few times by shaking back and forth. (make sure gel and QG buffer are at the bottom of tube when done.)

2. Incubate in **heating block @ 40°C** for 10-15 minutes (half way through shake tube back and forth with hand to mix again to help dissolve gel quicker) [make sure gel is dissolved, must be yellow in color to have proper pH. If **Pink,** adjust pH with 10 µl of 3M NaAc, pH5. *This rarely occurs.*].

3. Following the Qiagen protocol steps, add the ~750 µl sample to the Qiagen spin columns. Spin @ ~13000 rpm for 1 min. Dump waste from bottom waste tube. If sample is more than 750 µl, spin, dump, and repeat with remaining sample.

4. Add 750 µl of wash PE wash buffer to each spin column. **Let sit for 1 min.** Spin @ ~13000 rpm for 1 min. Dump waste from bottom waste tube.
   - Respin @ ~13000 rpm for 1 min. to dry spin column.

5. To elute DNA, put spin columns in new 1.5 ml tubes and **add 50 µl of warm (~40-50 °C)** EB (elution buffer). Reduce the EB volume used to 30 µl if the bands you cut out are faint. **Let sit 1 min.** then spin @ ~13000 rpm for 1 min.

6. QC 5 µl of each sample on gel with ladder & conc. standard to confirm size & quantity.

**Vector Ligation:**

1. In a clean, **well-labeled** PCR plate add **1 µl** of pUC18 SmaI/SAP vector (~100 ng) to each well. The pUC18 vector is from Roche and was cut (with SmaI) and prepared (dephosphorylated with SAP, and gel sized selected and purified) at the JGI

2. Transfer **10 µl** of the ~ 3 kb size selected gel purified BAC DNA sample (~200 ng) into a designated well of a 96 well PCR plate. Mix the DNA with the vector by mixing up and down gently a few times with the pipette.

3. In a 1.5 ml centrifuge tube on ice, prepare a cocktail mix of:

| | 1x | 15x |
|---|---|---|
| 10X Ligation Buffer (Fast-Link) per sample | 1.50 µl | 22.50 µl |
| 10mM ATP (Fast-Link) per sample --------- | 0.75 µl | 11.25 µl |
| Fast-Link DNA Ligase per sample ---------- | 2.00 µl | 30.00 µl |
| | **4.25** µl | 63.75 µl |

** Vortex well and quick spin tube before dispensing.

4. Dispense **4.25 µl** (use Matrix) of cocktail mix to each sample in the PCR plate (on the side wall of each well). Put plate in black base, **seal top**, & Mix/Vortex gently. Quick spin the plate.

5. In a PE 9700 PCR machine set up the following ligation protocol times. 24.5°C for 90 min, 70°C for 15 minutes, and a hold of 4°C. Reaction can stay overnight @ 4 °C if needed, but should be stored @ -20 °C for long term storage.

## Transformation - - Materials & Reagents

| Materials/Reagents/Equipment | Vendor | Stock Number |
|---|---|---|
| *Disposables* | | |
| Gene Pulse Cuvette 0.1 cm electrode gap | BioRad | 165-2089 |
| Falcon 14 mL Polypropylene Tube | Becton Dickinson | 352059 |
| Cryogenic Vial | Corning | 430289 |
| LB Amp 100 X-gal Plates | Teknova | L4902 |
| *Reagents* | | |
| ElectroMAX DH10B Cells (box of 5 x 100 µl ea.) | Invitrogen | 18290015 |
| SOC Medium | Teknova | 0166-10 |
| *Equipment* | | |
| Gene Pulser II | BioRad | |
| Pulse Controller Plus | BioRad | |

## Transformation—(Electro)

**Equipment Settings (BioRad Pulse Controller):**

- Low range: 200
- High range: ∞ (not used)
- Capacitance: 25
- Voltage: 1.8 kV

**Procedure:**

1. ***Place on ice: eppendorf tube and cuvette.***
2. Thaw ElectroMax DH10B competent cells on ice (each tube contains 100 µl, enough for 2 rxns).
3. To the appropriately **well-labeled**, COLD eppendorf tube; add **1 µl** of ligation product.
4. Once thawed, mix competent cells by **swirling** with pipette tip a few times
5. Add **50 µl** eDH10B competent cells to the eppendorf tube.
6. Mix by **swirling** ligation and competent cells together with pipette tip a few times

7. Transfer solution to the bottom groove of COLD cuvette and **tap** on tabletop a few times to settle solution to the bottom (**must see even levels of cells on each side of the cuvette without bubbles**).

8. Electroporate at 1.8 kV.

9. Transfer cell solution **IMMEDIATELY** to 950 µl of SOC **(make sure SOC is clear, i.e. no growth)**.
   *(Transfer electroporation within 10 seconds.)*

10. Rinse cuvette with 50 µl of the same SOC mixture you just added the cells to.

11. Incubate within rotating wheel at 37 °C for 1 hour.

12. After incubation, place on ice (no more than one hour) until ready to plate on agar plates.

**Plating:**

1. When 60 minute transformation incubation begins, prepare one **well-labeled** LB/Amp/IPTG/X-gal agar plate per library by letting them warm to 37°C in an incubator to dry upside down and open.

2. After 60 minute transformation incubation, make a 10% glycerol transformation stock (140 µl 80% glycerol + full transformation = ~ 1140 µl glycerol transformation stock). Cap, then mix by inverting several times.

3. Plate ~20 µl of transformation glycerol mixture onto the appropriately labeled bioassay.
   - First pipette ~ 500 µl SOC onto the center of the plate, then add 20 µl of transformation to the center with the SOC.
   - Spread in a **small** circular motion at first to help mix, and then spread evenly across the entire plate.

4. Store transformation glycerol mixture immediately @ -80 °C.

5. Incubate the plates upside down in 37°C incubator for ~18 hrs.

6. Count colonies and determine the complexity of ligation reaction (total # of colonies in ligation).

## PCR QC - - Materials & Reagents

| Materials/Reagents/Equipment | Vendor | Stock Number |
|---|---|---|
| *Disposables* | | |
| PCR plate | | |
| *Stock Solutions* | | |
| pUC-F primer | IDT | CTT TAC ACT TTA TGC TTC C |
| pUC-R primer | IDT | GCA AGG CGA TTA AGT TGG |
| *Reagents* | | |
| Taq | | |
| Buffer | | |
| dNTPs | | |
| *Equipment* | | |
| PE 9700 PCR machine | | |

## PCR for QC of library insert size

1. Set up a PE 9700 with the following colony PCR program: This is a **3 hr 15 min protocol**.

   94 °C – 4 min
   94 °C – 30 sec, 55 °C – 30 sec., 68 °C – *2 min. (35 cycles)
   (*modify with 5 sec. added on to each cycle for the 68 °C elongation step.)
   4 °C – Hold

2. Make up the following PCR master mix for each sample (x120 for a 96 well plate): Keep on ICE.

| | 1x | 120x |
|---|---|---|
| $H_2O$ ---------- | 16.85 µl | 2022.00 µl |
| 10x PCR Buffer ---------- | 2.00 µl | 240.00 µl |
| 10mM dNTP (Amersham)----- | 0.40 µl | 48.00 µl |
| pUC-F primer (10 pmol/µl)---- | 0.28 µl | 33.60 µl |
| pUC-R primer (10 pmol/µl) ---- | 0.28 µl | 33.60 µl |
| Taq (Amersham) ---------- | 0.28 µl | 33.60 µl |
| | **20.09** µl | 2410.8 µl |

3. Add **20 µl** of PCR mix into each well needed of the PCR plate. Keep plate on ice.

4. Using pipette tips only, pick desired number of colonies per library (usually 24) into their own well.

5. Pick 12 at a time then throw out tips. Be careful of your well location and of contamination.

6. Quick spin plate and place in PCR machine with above protocol.

7. After the 3.15 hrs run load 10 µl of the qc samples + 5 µl loading dye onto gel with proper size markers. Run for ~ 30 min @ 120 v.
   - Add 10 µl loading dye (must be weak colors so the PCR bands are not hidden) to PCR plate.
   - Mix before loading with up/down motion using pipette.
   - Load 15 µl.
   - Save the remaining 15 µl to use in case there is a problem with the QC gel. Can toss after gel is imaged.

## Reagent / Stock Preparation

1. 80% Glycerol Stock Solution
   - Use 100 mL glass bottle (autoclaved)
   - Add 40 mL of 100% glycerol to 10 mL of H20 (pipette slowly due to very viscous solution)
   - Autoclave.